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Oxidation Products of *β*-Carotene during the Peroxidation of Methyl Linoleate in the Bulk Phase

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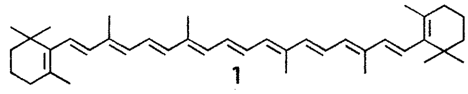
Methyl linoleate containing *β*-carotene was autoxidized or photooxidized at 37°C in the bulk phase, and the oxidation products of *β*-carotene were analyzed by high-performance liquid chromatography. Formyl *β*-carotenes, *β*-carotene 5,6-epoxide, and cyclic ethers of *β*-carotene were detected as the oxidation products during the peroxidation of methyl linoleate initiated by a free radical initiator. These products, which were also detected in the methyl linoleate autoxidized without an initiator, were detectable only in much smaller amounts than the consumed *β*-carotene. In the chlorophyll-sensitized photooxidation process, the products were *β*-carotene 5,8-endoperoxide and *β*-carotene 5,6-epoxide. *α*-Tocopherol partially inhibited the formation of the 5,6-epoxide, but had no effect on the main product, the 5,8-endoperoxide. These results indicate that *β*-carotene reacted with singlet oxygen to form the 5,8-endoperoxide as the primary product during the photooxidation of methyl linoleate, and that *β*-carotene trapped lipid-peroxyl radicals to form oxygenated products which decomposed immediately during the autoxidation process.

Key words: *β*-carotene; autoxidation; photooxidation; singlet oxygen; antioxidant



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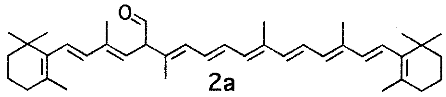

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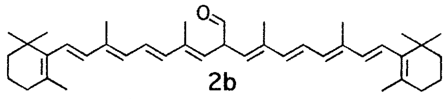

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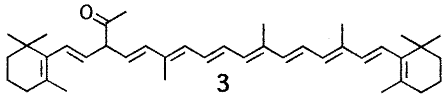

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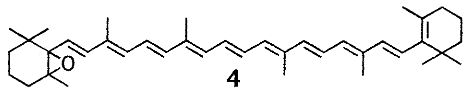

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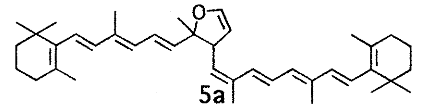

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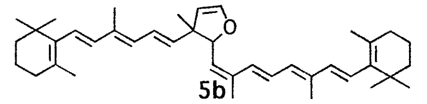

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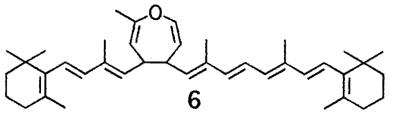

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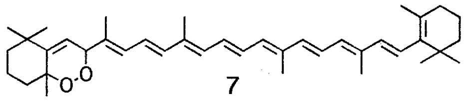

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β-Carotene (**1**) and other carotenoids exert an antioxidative function such as quenching singlet oxygen ($^1O_2$) and other electronically excited molecules that are produced by a photoexcitation or chemiexcitation reaction.[1,2] They also act as antioxidants by trapping chain-propagating peroxyl radicals.[3,4] It has been proposed that *β*-carotene acts as an antioxidant by scavenging lipid-peroxyl radicals, not by donating a hydrogen atom like *α*-tocopherol and ascorbic acid, but by an addition reaction to the double bond to give resonance-stabilized, carbon-centered, conjugated radicals.[4] Carotenoids have been demonstrated to inhibit the peroxyl radical-mediated oxidation of lipids in a solution,[5-7] of phospholipid liposomes,[7-9] of microsomal membranes,[10] and of *in vivo* systems.[11,12]

Knowledge of the fundamental chemistry of carotenoid radicals and of the reactions of carotenoids with oxidizing agents is important for evaluating the proposed action of carotenoids as free radical scavengers and $^1O_2$ quenchers. Thus, the oxidation products of *β*-carotene with peroxyl radicals generated by using azo compounds have been investigated.[7,13-17] In our previous study,[16] we isolated and characterized the reaction products of *β*-carotene with alkylperoxyl radicals in a benzene solution. The presence of long and conjugated double bonds makes *β*-carotene an excellent substance for free radical attack, and the resulting products are very complex. Similar chemical reactions also occur between *β*-carotene and $^1O_2$. The photosensitized oxidation of *β*-caro-

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Abbreviations: AMVN, 2,2′-azo*bis*(2,4-dimethylvaleronitrile); HPLC, high-performance liquid chromatography; MLOOH, methyl linoleate hydroperoxides; $^1O_2$, singlet oxygen; UV, ultraviolet tene has yielded β-carotene 5,8-endoperoxide and some chain cleavage products which were formed from the action of $^{1}O_{2}$.[18] However, the formation and the fate of these oxidation products during lipid peroxidation are still unclear.

This paper reports the formation of oxidation products of β-carotene during the autoxidation and chlorophyll-sensitized photooxidation of methyl linoleate in the bulk phase.

Materials and Methods

Materials. All-*trans*-β-carotene was obtained from Wako Pure Chemical Industries (Osaka, Japan) and recrystallized from benzene. Methyl linoleate was purchased from Tokyo Kasei Co. (Tokyo, Japan) and purified by silica-gel column chromatography to be peroxide-free.[19] Methyl linoleate hydroperoxides (MLOOH) were isolated from autoxidized or photooxidized methyl linoleate by silica-gel column chromatography.[20,21] *RRR*-α-Tocopherol from Sigma Chemical Co. (St. Louis, MO, U.S.A.) was purified by reversed-phase high-performance liquid chromatography (HPLC).[22] 2,2′-Azo*bis*(2,4-dimethylvaleronitrile) (AMVN) was obtained from Wako Pure Chemical Industries and used as received. Chlorophyll a was prepared from spinach leaves.[23]

Preparation of the standard oxidation products of β-carotene. The authentic oxidation products of β-carotene, a mixture of 12-formyl-11-nor-β,β-carotene (**2a**) and 15′-formyl-15-nor-β,β-carotene (**2b**), 19-oxomethyl-10-nor-β,β-carotene (**3**), 5,6-epoxy-5,6-dihydro-β,β-carotene (**4**, β-carotene 5,6-epoxide), a mixture of 13,15′-epoxyvinyleno-13,15′-dihydro-14,15-dinor-β,β-carotene (**5a**) and 15′,13-epoxyvinyleno-13,15′-dihydro-14,15-dinor-β,β-carotene (**5b**), and 11,15′-dihydrooxepin-β,β-carotene (**6**), were prepared from the peroxyl-radical oxidation of β-carotene by using the free radical initiator, AMVN, in benzene.[16] 5,8-Epidioxy-5,8-dihydro-β,β-carotene (**7**, β-carotene 5,8-endoperoxide) was obtained by the photosensitized oxidation of β-carotene according to the method described by Stratton *et al.*,[18] and its structure was confirmed by the following spectral data: UV-vis (hexane) λ_{max} nm (ε): 403 (91,700), 425 (106,000), 449 (72,600); IR ν_{max} (film) cm^{-1}: 2928, 2865, 1456, 1368, 1216, 1080, 966, 758; EIMS *m/z* (rel. int.) at 70 eV: 568 (M^{+}, 18%), 552 ([M—16]$^{+}$, 14), 472 (12), 404 (12), 221 (11), 205 (31), 165 (100), 119 (50), 105 (81); ^{1}H (270.17 MHz) NMR δ (CDCl$_3$): 1.03 (6H, s, H-16′,17′), 1.04 and 1.10 (3H, s, H-16), 1.12 and 1.17 (3H, s, H-17), 1.43–1.49 (4H, m, H-2,2′), 1.56–1.75 (4H, m, H-3,3′), 1.60 (3H, s, H-18), 1.71 (3H, s, H-18′), 1.89–2.08 (4H, m, H-4,4′), 1.92 (3H, s, H-19), 1.95 (3H, 4s, H-20), 1.97 (6H, 4s, H-19′,20′), 4.65 (1H, d, *J*=3.8 Hz, H-8), 5.56 (1H, d, *J*=3.8 Hz, H-7), 6.08–6.16 (4H, m, H-7′,8′,10,10′), 6.23 (2H, broad d, *J*=10.8 Hz, H-14,14′), 6.34 (1H, d, *J*=14.9 Hz, H-12), 6.35 (1H, d, *J*=14.9 Hz, H-12′), 6.52 (1H, dd, *J*=10.8, 14.9 Hz, H-11), 6.55–6.70 (3H, m, H-11′, 15,15′); ^{13}C (67.94 MHz) NMR δ (CDCl$_3$): 12.8 (3 atoms), 18.9, 19.3 (2 atoms), 21.8, 25.6, 27.8, 29.0 (2 atoms), 30.8, 33.1, 34.3, 35.4, 35.6, 39.7, 41.1, 79.8 (C-8), 83.0 (C-5), 116.1, 124.3, 125.1, 126.7, 128.9, 129.4, 129.9, 130.1, 130.8, 132.3, 132.5, 136.1, 136.1, 136.5, 137.2, 137.3, 137.8, 138.0, 138.1, 149.2.

Autoxidation. Methyl linoleate (1.0 g) containing β-carotene (0.05 mol%, based on methyl linoleate) with or without α-tocopherol (0.005 mol%, based on methyl linoleate) was placed into a glass vial (2.0 cm in diameter). The reaction mixture was incubated at 37°C in the dark under air in the presence or absence of AMVN (2 mol%, based on methyl linoleate). An aliquot of the sample was periodically withdrawn, dissolved in ethanol and injected into HPLC apparatus. The amount of MLOOH was determined by reversed-phase HPLC with ultraviolet (UV) detection,[24] α-tocopherol being quantified by HPLC with fluorescence detection.[25] β-Carotene and its oxidation products were analyzed after dissolving the sample in ethyl acetate and injecting into HPLC apparatus, using a Puresil 5 μC18 120 Å column (4.6 × 150 mm, Nihon Waters; Tokyo, Japan) with methanol–ethyl acetate (85:15, v/v) at 1.0 ml/min. The eluate was monitored by its absorbance at 340 or 450 nm. The amounts of β-carotene and its oxidation products were determined by their peak areas calibrated against known amounts of the authentic standards.

Photosensitized oxidation. Methyl linoleate (1.0 g) containing β-carotene (0.005 mol%, based on methyl linoleate) was placed in a glass vial (2.0 cm in diameter) with or without α-tocopherol (0.005 mol%, based on methyl linoleate), and illuminated with a 60-W tungsten lamp (at a distance of 30 cm from the sample) at 37°C under air. Chlorophyll a (0.005 mol%, based on methyl linoleate) was used as the photosensitizer. An aliquot of the sample was periodically withdrawn, dissolved in ethanol and analyzed by HPLC. The authentic standard of MLOOH used was prepared from the photosensitized oxidation already described. To analyze β-carotene and its oxidation products, chlorophyll a was removed by solid-phase extraction before the HPLC analysis. The sample was dissolved in 0.5 ml of benzene and the solution applied to a Sep-Pak silica cartridge (Waters; Milford, MA, U.S.A.). β-Carotene and its oxidation products were eluted with 4 ml of benzene. The eluted fractions were pooled and evaporated under nitrogen gas. The residue was dissolved in ethyl acetate and analyzed by HPLC under the same conditions as those just described.

Results

Oxidation products of β-carotene during the AMVN-initiated peroxidation and autoxidation of methyl linoleate

Methyl linoleate containing β-carotene was oxidized in the presence of AMVN at 37°C for 1 h, and the oxidation products of β-carotene were analyzed by reversed-phase HPLC (Fig. 1). Four product peaks, **2**, **4**, **5** and **6**, appeared on the chromatogram, along with several unknown peaks and a peak corresponding to β-carotene (**1**). The identity of each peak was confirmed by co-elu-

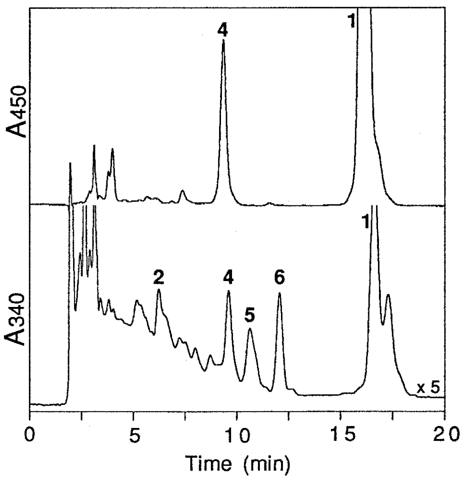

Fig. 1. Reversed-phase HPLC of the Oxidation Products of β-Carotene during the AMVN-initiated Peroxidation of Methyl Linoleate.

Methyl linoleate containing 0.05 mol% β-carotene was oxidized by the addition of 2 mol% AMVN at 37°C for 1 h. HPLC was conducted in a Puresil 5μC18 column (4.6 × 150 mm) developed with methanol-ethyl acetate (85:15, v/v) at 1.0 ml/min. The eluate was monitored by its absorbance at 340 or 450 nm, the sensitivity at 340 nm being 5 times higher than that at 450 nm.

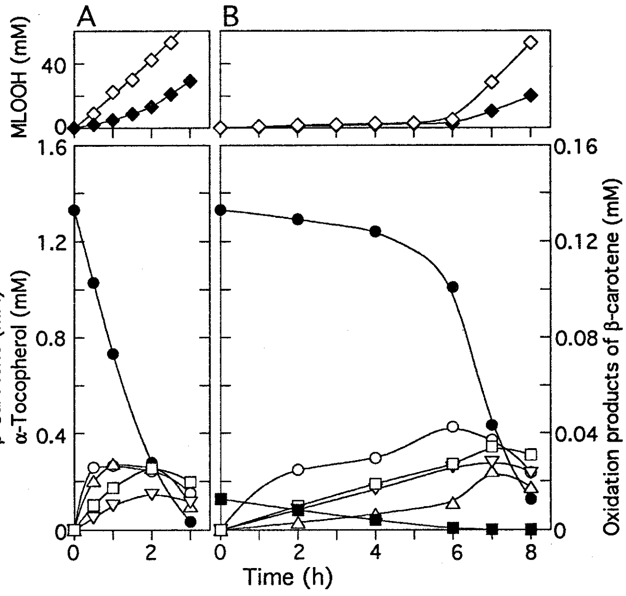

Fig. 2. Reaction of β-Carotene during the AMVN-Initiated Peroxidation of Methyl Linoleate.

Methyl linoleate containing 0.05 mol% β-carotene was oxidized at 37°C under air by the addition of 2 mol% AMVN. The reaction was carried out in the absence (A) or presence (B) of 0.005 mol% α-tocopherol. MLOOH without (◇) or with (◆) β-carotene; ● β-carotene; ○ compound 2; △ compound 4; ▽ compound 5; □ compound 6; ■ α-tocopherol.

tion against authentic standards[16] as follows: **2**, a mixture of 12-formyl-11-nor-β,β-carotene (**2a**) and 15′-formyl-15-nor-β,β-carotene (**2b**); **4**, β-carotene 5,6-epoxide; **5**, a mixture of 13,15′-epoxyvinyleno-13,15′-dihydro-14,15-dinor-β,β-carotene (**5a**) and 15′-13-epoxyvinyleno-13,15′-dihydro-14,15-dinor-β,β-carotene (**5b**);

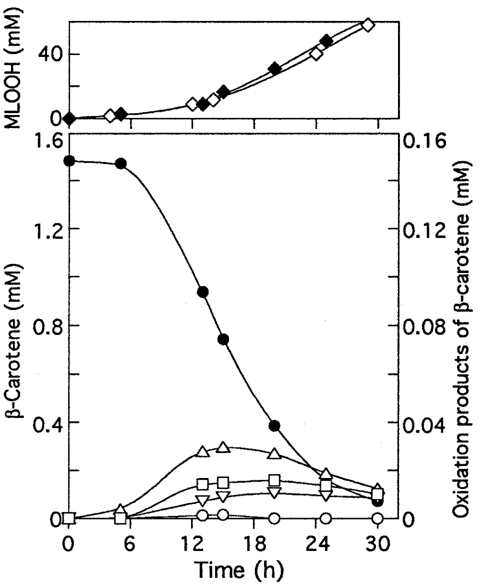

Fig. 3. Reaction of β-Carotene during the Autoxidation of Methyl Linoleate.

Methyl linoleate containing 0.05 mol% β-carotene was autoxidized at 37°C under air. MLOOH without (◇) or with (◆) β-carotene; ● β-carotene; ○ compound 2; △ compound 4; ▽ compound 5; □ compound 6.

and **6**, 11,15′-dihydrooxepin-β,β-carotene. The structure for compound **6** reported in our previous paper,[16] 11,15′-cyclo-12,15-epoxy-11,12,15,15′-tetrahydro-β,β-carotene, is incorrect. The revised structure, 11,15′-dihydrooxepin-β,β-carotene, has recently been reported by Zürcher *et al.*[26]

Figure 2 shows the results of the formation of MLOOH, the fate of β-carotene, and production of the oxidation products of β-carotene during the AMVN-initiated peroxidation of methyl linoleate. β-Carotene suppressed the formation of MLOOH, although it did not produce as clear an induction period as α-tocopherol did; α-tocopherol of much lower concentration suppressed the autoxidation almost completely until it was depleted.[7] Without α-tocopherol, β-carotene was rapidly oxidized, the oxidation products first formed being compounds **2** and **4**, after which compounds **5** and **6** appeared in the reaction mixture (Fig. 2A). α-Tocopherol retarded the disappearance of β-carotene and the formation of compound **4** until α-tocopherol had itself disappeared, but it did not inhibit the formation of compounds **2**, **5** and **6** (Fig. 2B). The oxidation products in the reaction mixture were detectable only in much smaller amounts than the amount of β-carotene that was destroyed.

When the peroxidation was carried out in the absence of AMVN, β-carotene did not suppress the formation of MLOOH (Fig. 3). The oxidation products of β-carotene produced were compounds **4**, **5** and **6**, whereas only a trace amount of **2** was detected during the autoxidation. We did not investigate the autoxidation process in the presence of α-tocopherol because α-tocopherol was a strong antioxidant and had a long induction period (da-

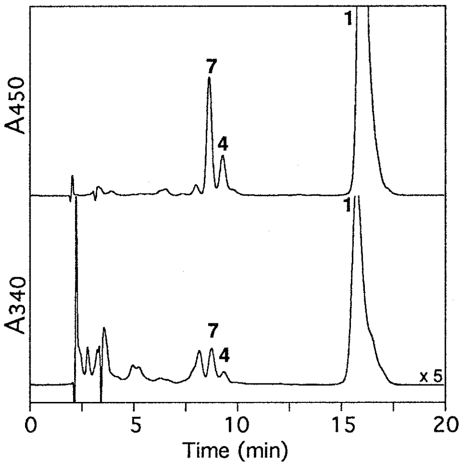

Fig. 4. Reversed-phase HPLC of the Oxidation Products of β-Carotene during the Chlorophyll-sensitized Photooxidation of Methyl Linoleate.

Methyl linoleate containing 0.005 mol% β-carotene and 0.005 mol% chlorophyll a was illuminated by a 60-W tungsten lump at 37°C for 3 h. The same HPLC conditions were employed as those in Fig. 1.

ta not shown).

Oxidation products of β-carotene during the chlorophyll-sensitized photooxidation of methyl linoleate

There is enough evidence to state that the chlorophyll-sensitized photooxidation of unsaturated lipids proceeds through $^1O_2$ oxygenation.[27,28] β-Carotene has inhibited $^1O_2$ oxygenation through physical quenching or a chemical reaction.[1] Although physical quenching plays a major part in the $^1O_2$ quenching activity of β-carotene, β-carotene is oxidized by a chemical reaction with $^1O_2$.[18] Figure 4 shows the oxidation products of β-carotene during the chlorophyll-sensitized photooxidation of methyl linoleate. Two product peaks, **4** and **7**, were detected on the chromatogram. Each of the peaks was confirmed by the co-elution of authentic standards, and identified as β-carotene 5,6-oxide (**4**) and β-carotene 5,8-endoperoxide (**7**), respectively.

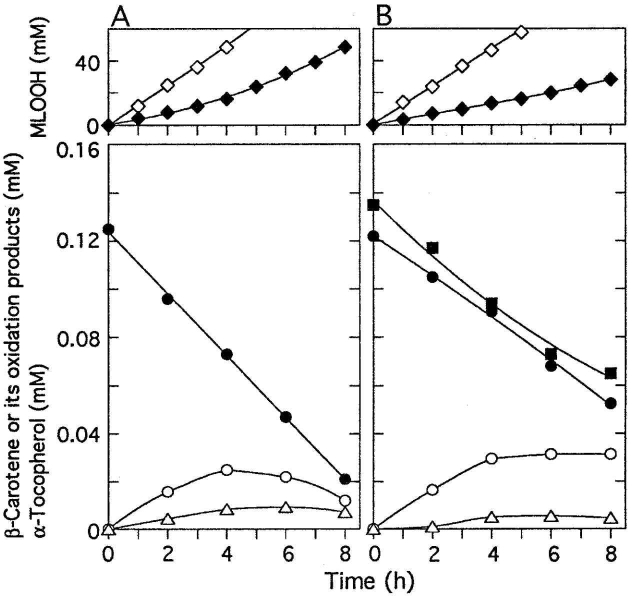

Fig. 5. Reaction of β-Carotene during the Chlorophyll-sensitized Photooxidation of Methyl Linoleate.

Methyl linoleate containing 0.005 mol% β-carotene was illuminated with a 60-W tungsten lump at 37°C. Chlorophyll a (0.005 mol%) was used as a photosensitizer. The photooxidation was carried out in the absence (A) and presence (B) of 0.005 mol% α-tocopherol. MLOOH without (◇) or with (◆) β-carotene; ● β-carotene; △ compound **4**; ○ compound **7**; ■ α-tocopherol.

Figure 5 shows the results of the chlorophyll-sensitized photooxidation of methyl linoleate in the presence of β-carotene. β-Carotene effectively retarded the forma-

Table 1. Relative Yields of β-Carotene and Its Oxidation Products during the Peroxidation of Methyl Linoleate in the Bulk Phase[a]

Incubation time (h)	Yield (%)[b]					
	β-Carotene	**2**	**4**	**5**	**6**	**7**
AMVN-initiated peroxidation						
1	55.0	2.0	2.1	0.8	1.3	
2	20.9	1.8	1.9	1.1	1.9	
+α-tocopherol						
2	97.0	1.9	0.2	0.6	0.7	
6	75.9	3.2	0.8	1.9	2.0	
Autoxidation						
13	63.2	0.1	1.9	0.5	1.0	
20	26.0	nd[c]	1.8	0.7	1.1	
Chlorophyll-sensitized photooxidation						
2	76.8		3.5			12.5
4	58.4		6.7			20.0
+α-tocopherol						
2	86.0		0.8			13.4
4	74.1		4.1			24.3

[a] These data are from the experiments described in Figs. 2, 3 and 5.
[b] Mol% to each theoretical yield is based on the amount of starting material.
[c] nd, not detectable.

tion of MLOOH, although the same concentration of α-tocopherol did not inhibit the photooxidation as efficiently as β-carotene did. The main oxidation product of β-carotene was compound **7**, and compounds **4** and **7** were accumulated with the decreasing amount of β-carotene (Fig. 5A). α-Tocopherol partially inhibited the formation of compound **4**, but it appeared to increase the yield of compound **7** (Fig. 5B).

Table 1 compares the relative yields of β-carotene products during the peroxidation of methyl linoleate. The oxidation products of β-carotene detected in the chlorophyll-sensitized photooxidation at an early stage accounted for almost all the consumed β-carotene, whereas the oxidation products in the autoxidation systems accounted for only a small part of the consumed β-carotene.

Discussion

β-Carotene has been proposed to exert antioxidative activity by a mechanism in which the chain-propagating peroxyl radical is trapped by addition to its conjugated polyene system.[4,9] This direct addition takes place to form a resonance-stabilized, carbon-centered radical which then reacts with a further peroxyl radical to form a non-radical product or reacts with oxygen to form a β-carotene-peroxyl radical.[7,9] The peroxyl-radical addition reaction of β-carotene in a solution has resulted in the formation of several oxygenated compounds, including carbonyl compounds, epoxides and cyclic ethers, as well as chain-cleavage products and polymeric products.[7,13-17] In this study, we detected aldehydes (**2a** and **2b**), a 5,6-epoxide (**4**), and cyclic ethers (**5a**, **5b** and **6**) as the oxidation products of β-carotene due to methyl linoleate peroxidation initiated by AMVN (Fig. 1). Thus, the reaction of the peroxyl radical addition to the polyene chain of β-carotene and subsequent intramolecular homolytic substitution (S_Hi) of the carbon-centered radicals might have occurred in this autoxidation system (Fig. 6).[16] Direct addition products of β-carotene and peroxyl radicals can be expected by this reaction mechanism. Liebler and McClure[29] have indicated direct evidence for the addition products being formed by the reaction of β-carotene with AMVN-derived free radicals by using atomospheric pressure chemical ionizing mass spectrometry. However, we could not detect such products under our experimental conditions.

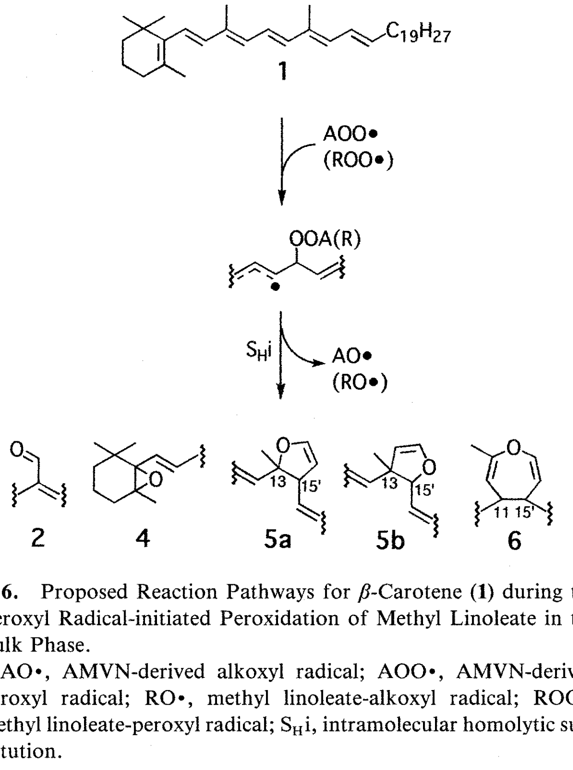

Fig. 6. Proposed Reaction Pathways for β-Carotene (**1**) during the Peroxyl Radical-initiated Peroxidation of Methyl Linoleate in the Bulk Phase.

AO•, AMVN-derived alkoxyl radical; AOO•, AMVN-derived peroxyl radical; RO•, methyl linoleate-alkoxyl radical; ROO•, methyl linoleate-peroxyl radical; S_Hi, intramolecular homolytic substitution.

β-Carotene is a weak antioxidant against peroxyl radical-initiated peroxidation when compared with α-tocopherol.[5,7,8] It has been reported that β-carotene was 32 times less reactive toward peroxyl radicals than α-tocopherol.[7] In the presence of both antioxidants, therefore, α-tocopherol predominantly scavenged peroxyl radicals and retarded both MLOOH formation and β-carotene depletion until α-tocopherol had disappeared (Fig. 2B). α-Tocopherol also inhibited the formation of oxidation products **4**, whereas it could not inhibit the formation of other product **2**, **5** and **6** (Fig. 2B). This indicates that a direct reaction between β-carotene and the AMVN-derived peroxyl radicals which could not be trapped by α-tocopherol might have produced compounds **2**, **5** and **6** and that α-tocopherol prevented the decomposition of these products. In contrast, α-tocopherol might have suppressed the S_Hi reaction of the carbon-centered radical to the 5,6-epoxide (**4**). However, the formation mechanism for these oxidation products during lipid autoxidation is still unclear. Further studies on β-carotene reactions would be necessary to confirm the antioxidative ability of β-carotene. The oxidation products of β-carotene were only formed after the propagation process had started in the autoxidation of methyl linoleate (Fig. 3). Therefore, the oxidation products of β-carotene would have been formed only in part during the initiation process, a more important route to these products probably being the chain propagating step in which a peroxyl radical adds to β-carotene before the S_Hi reaction to produce the products (Fig. 6).[17]

It is well known that carotenoids inhibit $^1O_2$ oxygenation through physical quenching and a chemical reaction.[1] β-Carotene reacts with $^1O_2$ by a chemical reaction, resulting in the loss of β-carotene and the formation of its oxidation products, including β-carotene 5,8-endoperoxide, apocarotenals and β-ionone.[18] We have confirmed the formation of β-carotene 5,8-endoperoxide (**7**) and β-carotene 5,6-epoxide (**4**) as oxidation products during the chlorophyll-sensitized photooxidation of methyl linoleate (Figs. 4 and 5). The result suggests that the product mixture from photooxidation is relatively simple and that two concurrent processes are at play. Since α-tocopherol did not inhibit the formation of compound **7**, this compound is assumed to have been the primary product of the reaction with $^1O_2$. The 5,8-endoperoxide could account for a large part of the consumed β-carotene in the early stage of photooxidation (Table 1). Therefore, 1,4-cycloaddition of $^1O_2$ with

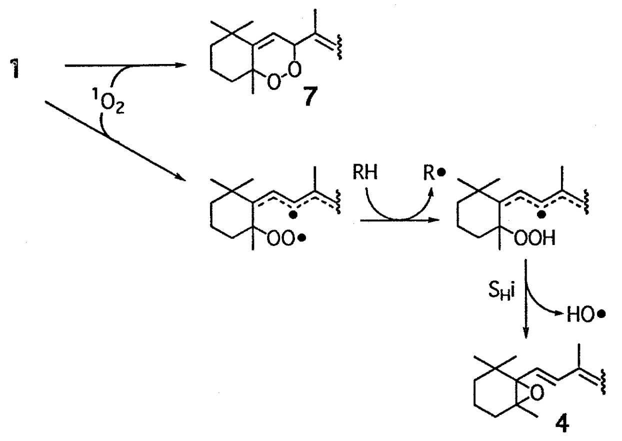

Fig. 7. Proposed Reaction Pathways for *β*-Carotene (**1**) during the Chlorophyll-sensitized Photooxidation of Methyl Linoleate in the Bulk Phase.

RH, methyl linoleate; S_Hi, intramolecular homolytic substitution.

β-carotene was the main chemical reaction in this system (Fig. 7). In addition, the ability of *α*-tocopherol to partially inhibit the formation of compound **4** (Fig. 5B) suggests that an oxygen-centered radical intermediate of *β*-carotene was involved in its formation. Clark *et al.*[30] have reported that an oxygen-centered radical of retinoic acid abstracted hydrogen from the substrate, resulting in a hydroperoxy carbon-centered radical which in turn would undergo an S_Hi reaction, yielding an epoxide. Thus, epoxide **4** might have been formed according to this mechanism in the initial steps of $^1O_2$ oxidation (Fig. 7). However, it is still unclear whether *α*-tocopherol inhibited the formation of the hydroperoxy carbon-centered radical or its decomposition to **4**.

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